Exhibit D

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Immunotherapy of Established Micrometastases with *Bacillus Calmette-Guérin* Tumor Cell Vaccine[1]

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ABSTRACT

We evaluated the use of *Bacillus Calmette-Guérin* admixed with tumor cells as a vaccine to induce systemic tumor immunity for therapy of subclinical (micrometastatic) disease. In several experiments inbred strain 2 guinea pigs were given i.v. injections of either 10^4, 10^5, or 10^6 syngeneic L10 hepatocarcinoma cells, and initial vaccinations were administered either 1 or 4 days after tumor inoculation. Variables in vaccine preparation, such as ratio of viable *Bacillus Calmette-Guérin* organisms to tumor cells, procedures for freezing the tumor cells, X-ray treatment of tumor cells, and vaccination regimen were evaluated. The studies demonstrated that under defined conditions nontumorigenic vaccines of *Bacillus Calmette-Guérin* and tumor cells can cure the majority of animals of otherwise lethal visceral micrometastases.

INTRODUCTION

The strategy of immunotherapy for cancer in experimental animal models and humans is limited by many factors including the stage, type, and location of the tumor; the level of antigenicity of the tumor cells; and the status of the host immune response. Clinical immunotherapy has been proceeding with relatively limited guidance from experimental animal models. Of the several approaches to immunotherapy of localized tumor and/or disseminated minimal residual tumor, immune potentiation by microbial agents has received the greatest attention. The most encouraging experimental and clinical data to date have resulted from protocols consisting of bacterial vaccines or nonspecific immunostimulants, primarily *Mycobacterium bovis* strain BCG,[2] administered i.t. (17, 18, 21) or systemically either alone (7, 8, 16) or admixed with tumor cells in the form of a vaccine (22, 23). One impetus for the use of BCG in immunotherapy has been the development of an experimental system that meets some of the requirements of a model to study an established tumor with regional lymph node metastasis (19). It has been demonstrated that regression of transplanted syngeneic hepatocarcinomas growing in the skin of inbred strain 2 guinea pigs and elimination of regional lymph node metastases are achieved in the majority of animals after i.t. injection of viable BCG (12, 26). This particular aspect of immunotherapy in the guinea pig model, although intriguing, is very limited with respect to the type, stage, and location of the tumor as well as with respect to the route of administration of BCG. Nevertheless, the initial studies established 1 fact that has broad implications. During BCG-mediated tumor regression and elimination of regional lymph node metastases, there is the development of systemic cell-mediated tumor immunity demonstrated by rapid rejection of a second tumor challenge several weeks after BCG treatment (11, 25, 27). This is a very important aspect of the model since it is known that, at the tumor stage when BCG administration is optimally effective, surgical excision of the tumor and regional lymph node would also be curative. However, no significant development of tumor immunity is achieved with surgery alone.

We recently demonstrated the effectiveness of tumor immunity induced by i.t. injections to eliminate artificially produced distant tumor foci (9, 10). This aspect of the BCG therapy model becomes important when one considers that adjuvant immunotherapy has been primarily tested in cancers for which control of primary tumors is available with surgery, radiotherapy, and/or chemotherapy, but where there is a substantial rate of relapse. Recurrence is usually thought to be due to a small number of residual tumor cells. Adjuvant immunotherapy is intended to eradicate the residual tumor cells by enhancing immunological mechanisms. However, based on all that we have learned, the translation to humans of the results of i.t. BCG injection in the guinea pig model would require careful attention to certain aspects of the treatment. These include tumor stage, dose, injection, route, regimen, and source of BCG. This is not always possible in human cancer, for which immunotherapy is often used for advanced cancer after other forms of treatment have failed. In addition, this model is inappropriate for cases in which i.t. injections are not possible.

An important advance in this guinea pig immunotherapy model would be to achieve effective systemic tumor immunity without the i.t. injection of BCG. We have approached this problem by systematically evaluating the ability of vaccines of BCG admixed with tumor cells to eliminate a disseminated tumor burden. Although previous attempts at BCG-tumor cell vaccine immunotherapy both in inbred guinea pigs (3) and in humans (see Ref. 20 for review) have been limited and somewhat discouraging, relatively little has been done to determine the optimal conditions for vaccination. Here we investigate a number of variables such as the ratio of viable BCG organisms to tumor cells, the freezing procedures, the X-ray treatment of cells, and the vaccination regimen. Although these factors cannot possibly be investigated systematically in humans for ethical reasons, they can be studied in the guinea pig model. Our studies demonstrate that, under defined conditions,

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[2] The abbreviations used are: BCG, *Bacillus Calmette-Guérin*; i.t., intratumoral; i.l., intralesional; L10, line 10 hepatocarcinoma cells; HBSS, Hanks' balanced salt solution; i.d., intradermal; PPD, purified protein derivative of *Bacillus Calmette-Guérin*.

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nontumorigenic vaccines of BCG and tumor cells can cure the majority of animals with lethal disseminated tumors established as visceral micrometastases.

MATERIALS AND METHODS

Animals. Inbred male Sewall Wright strain 2 guinea pigs were obtained from the Frederick Cancer Research Center Animal Breeding Section. These guinea pigs were shown to be histocompatible by skin grafting. They were housed 6 to 10 per cage and fed Wayne guinea pig chow and kale; they weighed 400 to 500 g at the beginning of the experiments.

Tumors. Induction of primary hepatocarcinomas in strain 2 guinea pigs after they were fed the water-soluble carcinogen, diethylnitrosamine, was described previously (19). The antigenic and biological properties of the transplantable ascites tumors developed from the primary hepatocarcinomas have also been described (28).

Ascites hepatocarcinoma cells, L10, were harvested and washed 3 times in HBSS and diluted to desired concentrations. One-ml doses of L10, ranging from 10^4 to 10^6 cells/dose, were injected into the dorsal vein of the penis, producing artificial vascular metastasis. Injections of 10^4 cells resulted in the death of approximately 70 to 80% of the animals, whereas 10^5 and 10^6 cells were fatal to all animals. The times of death varied as a function of dose, and all animals died as a result of metastasis to the lung, mediastinal lymph nodes, and hilar lymph nodes with concurrent visceral metastases.

BCG. *M. bovis* strain BCG (Phipps strain TNC 1029) was obtained from the Trudeau Institute (Saranac Lake, N. Y.). Preparations of BCG, stored at −70°, were rapidly thawed in a 37° water bath and diluted to proper concentrations.

Vaccine Preparation. The L10 tumor was maintained by i.p. passage in guinea pigs. Ascites cell preparations were removed and washed in HBSS. The L10 cells used in vaccine preparation were either fresh or frozen and thawed.

In preparation for freezing, the cells were concentrated and suspended in an equal volume of chilled 15% dimethyl sulfoxide plus 10% fetal calf serum-HBSS solution. The final suspension was 2 to 6 × 10^7 cells/ml. Two-ml aliquots of the L10 cell suspension were frozen at controlled rates in a Linde BF4 Biological Freezer at −1°/min to the critical freezing point, flash-frozen through the heat of fusion, and continued at −1°/min to a final temperature of −60°. The rate of freezing was monitored on a Honeywell Electronic III. The vials were stored in liquid nitrogen. The rationale for this method of freezing has been described in detail elsewhere (14, 15). The vials were rapidly thawed in a 37° water bath. Frozen-thawed cells were slowly diluted to 50 ml in HBSS, washed once, and resuspended in preparation for X-irradiation. Suspensions of fresh and frozen-thawed cells were X-irradiated in 50-ml beakers on ice. X-irradiation was performed with a Phillips MG 301 X-irradiation unit at 500 R/min. A total X-irradiation dose of 20,000 R was achieved. Cell viability counts were performed with the use of the trypan blue dye exclusion test, and viability after irradiation of either fresh or frozen-thawed cells was generally 90%, with less than 10% variation between the fresh or frozen-thawed cells.

BCG (10^9 organisms/ml) was added in equal volume to viable L10 (10^8 cells/ml) for a vaccine ratio of 10:1. A vaccination consisted of an i.d. injection of 0.2 ml. For ratios of 1:10, BCG (10^9 organisms/ml) was diluted 1:100 in HBSS, and aliquots were mixed with 10^8 viable L10 cells/ml. These vaccinations also consisted of an i.d. injection of 0.2 ml. All vaccinations were performed less that 1 hr after the BCG-tumor cell mixtures were prepared.

In preliminary vaccination experiments, the L10 cells were irradiated with 12,000 R; however, we noticed that, although this irradiated cell preparation was not tumorigenic when admixed with BCG, it was tumorigenic when administered i.d. in the absence of BCG. We were concerned that any growth of 12,000-R X-irradiated L10 cells in the skin might preempt developing tumor immunity and thus render the treatment ineffective against disseminated tumor. Therefore, 20,000-R X-irradiation was used in all subsequent experiments with L10 cells in BCG-tumor cell vaccines. Animals were given i.v. injections, in the dorsal vein of the penis, of either 10^4, 10^5, or 10^6 L10 cells in 1-ml volumes. All vaccinations were given i.d., beginning in the upper right dorsal quadrant. Successive vaccinations were given in different sites or i.l. in the previous vaccination site. Vaccinations were performed either 1 and 7 days or 4 and 10 days after i.v. L10 injection.

RESULTS

An i.v. dose of 10^4 L10 tumor cells does not lead to the death of all guinea pigs. Approximately 25% of the animals will survive clean injections where leakage did not occur to the regional site. This inoculum is the optimal dose for assessing the influence of the nonspecific side effects of vaccination on tumor cell arrest, the extravasation and establishment in organs, and the immunologically specific effects of the vaccine. Thus, at this initial tumor cell dose of 10^4, vaccinations were performed at either 1 and 7 or 4 and 10 days after i.v. injections of L10.

Several modes of vaccination as well as 2 ratios of viable BCG to tumor cells were tested in guinea pigs given i.v. injections of 10^4 L10 cells. The BCG-tumor cell ratios were 10^8 BCG or 10^6 BCG admixed with 10^7 L10. These were administered as either a single vaccination, a single injection of BCG-L10 vaccine followed 6 days later by an i.l. injection of L10 into the previous vaccination site, a single injection of BCG-L10 vaccine followed 6 days later by an injection of L10 alone on the opposite side, or 2 separate injections of BCG-L10 vaccine. Also, the efficacy of frozen L10 cells was compared to that of fresh L10 cells. The results are shown in Table 1.

Compared to the untreated tumor-bearing guinea pigs, no significant difference in survival was detected in animals treated with 2 i.d. injections of BCG or tumor cells alone, regardless of whether the initial treatment was performed 1 or 4 days after i.v. injection of L10.

Single BCG + L10 vaccinations at ratios of 1:10 or 10:1 did not confer significantly greater protection than did vaccinations of BCG alone, tumor cells alone, or nontreated controls. Furthermore, these 2 BCG:L10 ratios could not be associated with significant differences in survival of animals given i.v. injections of 10^4 tumor cells, regardless

Table 1

Survival of guinea pigs given i.v. injections of 10^4 syngeneic L10 hepatocarcinoma cells

This experiment was terminated at 280 days after tumor injection; all nontreated controls died by 120 days. Significance of differences in survival was calculated by the Fisher 2-tailed exact test

Treatment[a]	No. of survivors/total no. of animals/group at following vaccination times after i.v. injection of tumor	
	Days 1 and 7	Days 4 and 10
None	3/12	
(10^8 BCG) (10^8 BCG)	3/12	3/10
(10^7 L10) (10^7 L10)	2/10	
(10^6 BCG + 10^7 L10)[b]	4/10	4/10
(10^8 BCG + 10^7 L10)[b]	2/10	
(10^6 BCG + 10^7 L10) (10^7 L10 i.l.)	8/10	8/10
(10^8 BCG + 10^7 L10) (10^7 L10 i.l.)	9/10	9/10
(10^8 BCG + 10^7 FL10[c]) (10^7 FL10 i.l.)	8/10	
(10^6 BCG + 10^7 L10) (10^7 L10)	10/10	10/10
(10^8 BCG + 10^7 L10) (10^7 L10)	10/10	9/10
(10^6 BCG + 10^7 L10) (10^6 BCG + 10^7 L10)	10/10	10/10
(10^8 BCG + 10^7 L10) (10^8 BCG + 10^7 L10)	9/10	10/10

[a] Treatments were administered 6 days apart on opposite sides as described in "Materials and Methods."
[b] Vaccinations were administered as single sequential injections.
[c] FL10, frozen-thawed L10.

of the vaccination schedule. Compared to those animals that received single vaccinations of BCG + L10, BCG, or tumor cells alone and compared to the nontreated controls, significant differences in survival were achieved in tumor-bearing guinea pigs that received the second vaccination of either L10 i.l. ($p < 0.03$), L10 on the opposite side ($p < 0.01$), or BCG-L10 mixture ($p < 0.01$). From 80 to 100% of the animals survived in these treatment groups, regardless of whether the initial vaccine was administered 1 or 4 days after i.v. L10 injection. No significant differences in efficacy were detected between fresh L10 cells and frozen L10 cells.

At 280 days, representative groups of the survivors either were tested for tumor immunity by measurement of rejection of i.d. challenge of 10^6 L10 cells or were killed and autopsied for gross and histological examination for residual tumor. None of the animals autopsied had any evidence of residual tumor. Tumor challenge groups varied in their ability to reject contralateral challenge as a function of treatment. All nontreated controls or groups that had been treated with BCG or tumor cells alone failed to reject contralateral challenge, indicating that these animals were not tumor immune at 280 days after treatment. Seventy to 90% of the survivors in the various multiple vaccination groups rejected contralateral challenge; however, no significant difference in tumor immunity, as measured by contralateral challenge, could be detected among these treatment groups. These data demonstrate that animals that survived after treatment with ineffective modes of vaccination were not tumor immune, whereas significant protection as well as long-term tumor immunity was conferred on those animals that received efficacious modes of vaccination.

Injections of 10^5 or 10^6 syngeneic L10 cells i.v. are routinely fatal in strain 2 guinea pigs. Vaccinations of BCG alone or tumor cell alone conferred no protection in these tumor-bearing guinea pigs when the animals were given vaccinations 1 and 7 days after i.v. tumor inoculation (Table 2). Survival in all treatment groups was a function of the BCG:L10 cell ratio. Without exception, in guinea pigs given 10^5 or 10^6 cells i.v., a vaccine containing BCG:L10 cells in a ratio of 10:1 yielded significant protection, whereas a ratio of 1:10 was ineffective. Thus, the ratio of viable BCG organisms to tumor cells is a critical factor in the efficacy of the vaccine, and a large amount of BCG is beneficial in the initial vaccination. No significant difference in protection could be detected when the group that received a single BCG + L10 vaccination (10:1) was compared to a similar treatment group that received a second i.l. L10 injection. In contrast, survival was achieved in those animals that received a second injection of L10 alone or BCG + L10 on the opposite side ($p < 0.02$ or $p < 0.01$, respectively). In 2 groups of animals given i.v. injections of 10^6 L10, no significant difference in protection was detected when frozen-thawed L10 was used in the vaccine in place of the fresh L10.

One important consideration was whether BCG-immune guinea pigs could generate effective tumor immunity after

Table 2

Survival of guinea pigs given i.v. injections of 10^5 or 10^6 syngeneic L10 hepatocarcinoma cells

These experiments were terminated at 240 days after tumor injection. All nontreated controls in the 10^5 group died by 95 days, and all nontreated controls in the 10^6 group died by 77 days. Significance of differences in survival was calculated by the Fisher 2-tailed exact test.

Treatment[a]	No. of survivors/total no. of animals/group at following i.v. tumor cell dose	
	10^5	10^6
None	0/10	0/10
(10^8 BCG) (10^8 BCG)	0/10	0/10
(10^7 L10) (10^7 L10)	0/10	0/10
(10^6 BCG + 10^7 L10)[b]	1/10	0/10
(10^8 BCG + 10^7 L10)[b]	2/10	0/10
(10^6 BCG + 10^7 L10) (10^7 L10 i.l.)	1/10	
(10^8 BCG + 10^7 L10) (10^7 L10 i.l.)	5/10	
(10^6 BCG + 10^7 FL10[c]) (10^7 L10 FL10 i.l.)	5/10	
(10^6 BCG + 10^7 L10) (10^7 L10)	1/10	0/10
(10^8 BCG + 10^7 L10) (10^7 L10)	8/10	3/10
(10^6 BCG + 10^7 L10) (10^6 BCG + 10^7 L10)	1/10	1/10
(10^8 BCG + 10^7 L10) (10^8 BCG + 10^7 L10)	9/10	8/10
(10^6 BCG + 10^7 FL10) (10^6 BCG + 10^7 FL10)	9/10	

[a] Treatments were administered 6 days apart on opposite sides as described in "Materials and Methods."
[b] Vaccinations were administered as single sequential injections.
[c] FL10, frozen-thawed L10.

BCG + L10 vaccination. Normal guinea pigs as well as guinea pigs previously immunized to BCG and shown to be PPD positive by skin testing were given i.v. injections of 10^5 L10 cells. In this particular experiment vaccinations were performed 4 and 10 days after i.v. tumor inoculation. Two modes of vaccination, BCG + L10 (10:1) at Days 4 and 10 and BCG + L10 (10:1) at Day 4 followed by i.l. L10 at Day 10, were compared in PPD-positive and PPD-negative guinea pigs. Regardless of whether or not the animals were PPD positive, the 2 modes of vaccination conferred significant protection ($p < 0.01$) and did not differ significantly (Table 3).

We next investigated whether multiple BCG + L10 vaccinations at either the 10:1 or 1:10 ratios would improve survival in comparison to single or sequential vaccinations. Guinea pigs were given i.v. injections of 10^6 L10 cells and vaccinated on either 1 day, 1 and 7 days, or 1, 7, and 14 days after i.v. tumor inoculation. Treatments consisted of a single vaccination of BCG + L10 followed by L10 alone or by BCG + L10, 2 simultaneous BCG + L10 vaccinations followed by 2 simultaneous injections of L10 alone or of BCG + L10, or 3 sequential vaccinations of BCG + L10. The results are shown in Table 4.

Initial BCG + L10 vaccinations at a ratio of 1:10 were ineffective regardless of the vaccination schedule. Significant protection ($p < 0.01$) was achieved with all initial vaccinations at ratios of 10:1, but no significant increase in survival was achieved by multiple or sequential vaccinations.

DISCUSSION

Vaccines consisting of tumor cells admixed with BCG, under certain defined conditions, are effective in controlling and eliminating micrometastases in a syngeneic guinea pig tumor system, regardless of whether the animals are BCG immune or not. At the outset it should be stated that this experimental model has major limitations in that it is a transplantable tumor established for a short time in normal guinea pigs and in that the system out of necessity does not take into account such factors as individual variations between the biological behavior of tumors of other histological types and the limiting factors of the host. Thus, the model may be used to answer only specific questions fundamental to immunotherapy of micrometastasis.

Under natural conditions the development of metastasis is dependent upon an interplay between properties of the host and properties of the tumor cells. The process is highly selective and represents the end point of several destructive events from which few tumor cells survive. Only a few tumor cells within the primary neoplasm may actually invade blood vessels, and of those even fewer will survive in the circulation. Similarly, not all malignant cells that survive transport are successfully arrested, undergo extravasation, etc. Also, tumor cells, in principle, could be susceptible to host immune and nonimmune defense mechanisms that could destroy malignant cells during any of the steps described above (5, 6).

Metastasis was artificially induced in guinea pigs by i.v. injection of L10, and treatment was not started until adequate time had elapsed to ensure extravasation and localization of tumor cells into the parenchyma of visceral organs. No significant difference in the effectiveness of vaccines was found when the treatment was started 1 or 4 days after tumor cell transplantation. It has previously been demonstrated with i.v. injection of B16 melanoma in mice (4) that, between 1 and 4 hr after i.v. transplantation, there is a 50% reduction in the number of arrested tumor cells in the lung, and at 24 hr only 2% of the cells are retained in the lung as a stable metastatic population. Thus, the results of any treatment administered prior to 24 hr after transplantation are impossible to interpret since beneficial effects could be due to prevention of metastasis rather than to treatment. In this study the lack of difference between

Table 3

Survival of guinea pigs given i.v. injections of 10^5 syngeneic L10 hepatocarcinoma cells: effectiveness of vaccination in BCG-immune guinea pigs

Guinea pigs were given i.d. injections of 10^6 BCG and skin tested with PPD 21 days after immunization; 2 weeks later animals were given i.v. injections of 10^5 L10. The experiment was terminated 270 days after tumor injection, and all nontreated controls died by 128 days after tumor injection. Significance of differences in survival was calculated by the Fisher 2-tailed exact test.

PPD sensitivity	Treatment at 4 and 10 days	Survivors/total no. of animals/group
−	None	0
+	None	0
−	(10^6 BCG + 10^7 L10) (10^6 BCG + 10^7 L10)	5/10
+	(10^6 BCG + 10^7 L10) (10^6 BCG + 10^7 L10)	6/10
−	(10^6 BCG + 10^7 L10) (10^7 L10 i.l.)	2/10
+	(10^6 BCG + 10^7 L10) (10^7 L10 i.l.)	6/10

Table 4

Survival of guinea pigs given i.v. injections of 10^5 syngeneic L10 hepatocarcinoma cells: effect of multiple vaccinations

This experiment was evaluated at 120 days after i.v. tumor injection, and all animals in the nontreated or single vaccination control groups died by 70 days. Significance of differences in survival was calculated by the Fisher 2-tailed exact test.

Treatment[a]	Survivors/total no. of animals/group
None	0/13
(10^6 BCG + 10^7 L10)[b]	0/10
(10^6 BCG + 10^7 L10)[b]	0/10
(10^6 BCG + 10^7 L10) (10^7 L10)	0/10
2(10^6 BCG + 10^7 L10) 2(10^7 L10)[c]	1/9
(10^6 BCG + 10^7 L10) (10^7 L10)	3/10
2(10^6 BCG + 10^7 L10) 2(10^7 L10)	6/10
(10^6 BCG + 10^7 L10) (10^6 BCG + 10^7 L10)	1/10
2(10^6 BCG + 10^7 L10) 2(10^6 BCG + 10^7 L10)	1/10
(10^6 BCG + 10^7 L10) (10^6 BCG + 10^7 L10)	5/10
2(10^6 BCG + 10^7 L10) 2(10^6 BCG + 10^7 L10)	6/10
(10^6 BCG + 10^7 L10) (10^6 BCG + 10^7 L10) (10^6 BCG + 10^7 L10)	4/9

[a] Treatments were administered 6 days apart on opposite sides, as described in "Materials and Methods."
[b] Vaccinations were administered as single injections.
[c] Two simultaneous injections.

various effective vaccines when treatment was adminstered 1 or 4 days after transplantation suggests that a therapeutic effect was indeed achieved with these vaccinations.

It was clear from this study that, of the 2 basic BCG-tumor cell vaccines used, the preparation consisting of 10^6 viable BCG admixed with 10^7 tumor cells was more effective over a broad range of increasing initial tumor burdens than was that of 10^6 BCG admixed with 10^7 tumor cells. Although the latter was effective at initial tumor burdens at 10^4 L10, it was ineffective when the initial tumor cell inoculum was increased to 10^5 L10. Whether this 10:1 BCG:tumor cell ratio is critical or simply a function of total BCG cannot be determined from these studies. However, in a study of the effectiveness of BCG-tumor cell mixtures as vaccines against LSTRA murine leukemia (2), it was found that immunity was high (100%) if the BCG:LSTRA ratio was low (either 5×10^4:10^5 or 5×10^6:10^5) and that the proportion of immune mice was low (8%) if the BCG:LSTRA ratio was high (5×10^6:10^5).

Tumor cells that were frozen by an established procedure used for preservation of bone marrow in transplantation studies and assessed as an optimal procedure in several low-temperature biology studies (for review, see Ref. 24) were equally as effective in the vaccines as fresh tumor cells. This is contrary to the results of Bartlett *et al.* (1) who used glycerol as the freezing additive. Our cells were frozen in dimethyl sulfoxide and fetal calf serum. The striking difference, however, was the percentage of viability after freezing. Cell viability was approximately 90% after freeze-thawing. If for some reason viability fell below 80% during liquid nitrogen storage, the cells were discarded. In our opinion, the trypan blue exclusion test is a very conservative test of cell damage, and any trauma sufficient to render 20% of the cells sensitive to trypan blue may have severely damaged the remaining cells or altered their antigenicity. The viability of frozen cells in the experiments of Bartlett *et al.* ranged between 40 and 70% as determined by trypan blue exclusion. Thus, the difference in results with frozen L10 cells may be attributed to suboptimal *versus* optimal freezing conditions. Whether the 20,000-R X-irradiation dose of the tumor cells was an important aspect in the preparation of cells in vaccines is not known. However, studies are under way to test this point in this model since it is recognized that the use of 12,000 R is standard procedure for BCG-tumor cell vaccines in humans.

Of the 3 basic vaccination schedules tested, the 2 that were consistently effective for all tumor burdens were 10^6 BCG admixed with 10^7 L10 followed by 10^7 alone on the opposite side or 2 separate injections of 10^6 BCG admixed with 10^7 L10. The fact that BCG was not required in the second injection of the former schedule and the fact that multiple vaccinations did not improve therapy with respect to the latter schedule or with the less effective vaccine (10^6 BCG + 10^7 L10) suggest that the critical aspect of any of the vaccination schedules is the initial dose of BCG. Following the initial treatment with BCG, effective systemic tumor immunity can be achieved with i.d. injections of tumor cells alone at a different site.

A third vaccination schedule, which consisted of reintroduction of the tumor immunogen into the i.d. site previously injected with BCG + tumor cells, was effective at lower tumor burdens (10^4) but was less effective at initial tumor burdens of 10^5. This is in contrast to a similar schedule in which the second tumor cell vaccination was in a different i.d. site. The rationale for the second injection of tumor cells in the BCG-infected site was based on the study of Hawrylko (13), in which the dimensions of BCG-potentiated antitumor response against the murine mastocytoma P815 were investigated. One limitation that we found with this procedure was the difficulty in delivering the tumor cell inoculum in the previously infected dermal site. Early ulcerations of these injected dermal sites were limiting with respect to constant delivery of the tumor immunogen in the second injection.

In this study we have shown that visceral micrometastasis induced by i.v. injection of L10 can be cured by the systemic effect of a tumor cell-BCG vaccine. These results confirm our previous studies on the immunological susceptibility of i.v.-injected L10 cells (9, 10). We have now demonstrated that a nontumorigenic vaccine can affect immunotherapy. These results demonstrate that there is a critical dose for BCG in the initial vaccination but that BCG is not essential in the subsequent vaccination and that optimum therapy could be achieved with 2 vaccinations separated by a period of 6 days. Furthermore, the induced tumor immunity, which can cure the majority of guinea pigs with micrometastases is achieved by 2 vaccinations that require a total of 2×10^7 tumor cells (approximately 20 mg of tumor) administered over a period of 1 week. Also, the tumor cells, when frozen under established optimal conditions, maintain immunogenicity and can be used effectively in vaccines.

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